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Award Number: W81XWH-04-1-0612

TITLE: Identification of Genes Involved in Breast Tumor Invasion Utilizing a Ubiquitin-Mediated Proteolysis In Vitro Screen

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REPORT DATE: October 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20060503022

REPORT DOCUMENTATION PAGE

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-10-2005	Annual	30 Sep 2004 –29 Sep2005

4. TITLE AND SUBTITLE

Identification of Genes Involved in Breast Tumor Invasion Utilizing a Ubiquitin--Mediated Proteolysis In Vitro Screen

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04- Identification of

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Sidney Kimmel Cancer Center
San Diego, CA 92121

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In this proposal, we explore the potential use of ubiquitin-dependent proteolysis as a "reverse genetics" tool in functional genomics studies. We will develop a retroviral-based system that artificially targets random cellular proteins to the proteolytic machinery for degradation. To achieve this, a randomized peptide library will be linked to a segment of the F-box motif of beta-TrCP, the F-box protein that mediates the ubiquitination of I(kappa)B(alpha) and (beta)-catenin via the multimeric SCF ubiquitin ligase. The resultant chimeric proteins are expected to direct any interacting proteins that are otherwise stable, to the SCF ligase for ubiquitination. As proof of principle, we will use this system in a loss-of-function *in vitro* assay to identify putative genes involved in breast tumor invasion. MDA-MB-231 breast tumor-derived cells will be tranduced with the retroviral chimera library and peptides that confer the ability to invade through an artificial extracellular matrix will be isolated using a modified Boyden chamber assay. Following multiple rounds of selection, the chimeras that provide invasion properties will be confirmed using tumorgenicity assays in nude mice. If successful, ubiquitination-based functional assays will undoubtedly contribute to the identification of potential protein targets for therapeutic intervention in breast cancer.

15. SUBJECT TERMS
Invasion, Ubiquitin, Proteolysis, SCF, F-box, Functional Genomics

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	5	19b. TELEPHONE NUMBER (include area code)

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A. Introduction

The goal of this research project was to establish a functional genomics screen that utilizes a cell's own ubiquitin-dependent proteolysis system to screen for proteins involved in breast tumor invasion. The experimental design of the project was to express a randomized peptide library attached to a segment of the F-box domain of the protein β-TrCP in MDA-MB-231 breast tumor cells. The chimeric proteins are expected to bind to and recruit otherwise stable cellular proteins to the SCF ubiquitin ligase for ubiquitin-dependent degradation. Cells that express this construct would then be selected for a gain of invasion function using a Boyden chamber assay.

B. Body

We have asked for and were granted a one-year extension for the completion of this research project from the DOD Breast Cancer Research Program. The extension was requested based on unexpected complications in constructing and amplifying the proteolysis targeting vector library of sufficient molecular complexity for functional genomics experiments. This problem was due to an inefficient transformation efficiency of *E. coli* cells which has since been overcome (described below).

We have amplified a segment of the protein β-TrCP pertaining to the F-box domain from expression plasmid pFlag-CMV2-β-TrCP using the Advantage HF2 high fidelity polymerase (Clontech). We designed the oligonucleotides to include a 5' Flag epitope tag and 5' and 3' cloning sites for directional cloning. We sequenced the amplified DNA segment and found that it to be free of mutations (completion of point #1 of SOW).

We then devised an experimental strategy to generate the DNA segment that encodes the random peptide library. This strategy was based on annealing a degenerate oligonucleotide with a complementary anchor oligonucleotide (both synthesized by Sigma-Genosys). The oligonucleotides were designed to contain 5' and 3' restriction endonuclease sites for directional cloning, sequence which encodes a 5' poly-glycine linker sequence, and a degenerate $(N)^{21}$ DNA sequence that encodes peptides of random 7 amino acids. The sequence content of the degenerate oligonucleotide was designed to minimize the possibility of the introduction of a stop codon in the peptide library. We annealed the degenerate and anchor oligonucleotides and constructed the double-stranded DNA peptide library cassette by incubation with Klenow polymerase (Clontech). The efficiency of the annealing and synthesis reactions was evaluated by agarose gel electrophoresis (completion of SOW point #2).

We constructed the retroviral-based proteolysis targeting vector by first cloning the β-TrCP F-box segment synthesized above into the retroviral vector pLPC (a previous gift from S. Lowe, Cold Spring Harbor Laboratory). This vector was chosen due to its high level of expression from a CMV minimal promoter. We then directionally cloned the random peptide library cassette synthesized above in the cloning site 3' of the β-TrCP cassette. The efficiency of the ligation reaction was evaluated by agarose gel electrophoresis (completion of point #3 of SOW)

In order to perform a comprehensive functional genetic screen for genes involved in breast tumor invasion, we estimated that a molecular complexity of $>10^{10}$ different proteolysis targeting plasmids would be required. However, we encountered a "bottle-neck" problem in the amplification of the ligated proteolysis targeting vector. Using various commercially available strains of chemically competent *E. coli*, we could not

achieve a transformation frequency $>10^7/\mu g$ for the proteolysis targeting construct. Over the last 2-3 months, we have compared various commercially available chemically and electrocompetent strains of *E. coli* to optimize the transformation efficiency. We have now optimized our transformation procedure using a commercially available electrocompetent strain of *E. coli* that provides a transformation efficiency $>10^{10}/\mu g$ proteolysis targeting vector. This transformation efficiency avoids the "bottle-neck" on molecular complexity encountered in proteolysis targeting vector amplification and will allow for us complete the remaining points outlined in the SOW in the coming year.

C. Key research Accomplishments
Construction of a retroviral-based proteolysis targeting vector for expression of 7mer random peptides linked to a β-TrCP F-box domain.

D. Reportable Outcomes
Abstract: Tan, YM, Spruck, C. Identification of genes involved in breast tumor invasion utilizing an ubiquitin-mediated proteolysis in vitro screen. Era of Hope meeting 2005, sponsored by the DOD Breast Cancer Research Program

E. Conclusions
A one-year extension was asked for and granted from the DOD Breast Cancer Research Program for the completion of this research. The estimated completion date of the project is November 2006.

G. References
None

H. Appendices
None